PCT/AU2004/000494

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Third day of September 2004

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AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

Invention Title: **Manipulation of condensed tannin biosynthesis**

The invention is described in the following statement:

MANIPULATION OF CONDENSED TANNIN BIOSYNTHESIS

The present invention relates to nucleic acid fragments encoding amino acid sequences for flavonoid biosynthetic enzymes in plants, and the use thereof for the modification of flavonoid biosynthesis in plants, and more specifically the modification of the content of condensed tannins.

Flavonoids constitute a relatively diverse family of aromatic molecules that are derived from phenylalanine and malonyl-coenzyme A (CoA, via the fatty acid pathway). These compounds include six major subgroups that are found in most higher plants: the chalcones, flavones, flavonols, flavandiols, anthocyanins and condensed tannins (or proanthocyanidins). A seventh group, the aurones, is widespread, but not ubiquitous.

Some plant species also synthesize specialised forms of flavonoids, such as the isoflavonoids that are found in legumes and a small number of non-legume plants. Similarly, sorghum, maize and gloxinia are among the few species known to synthesize 3-deoxyanthocyanins (or phlobaphenes in the polymerised form). The stilbenes, which are closely related to flavonoids, are synthesised by another group of unrelated species that includes grape, peanut and pine.

Besides providing pigmentation to flowers, fruits, seeds, and leaves, flavonoids also have key roles in signalling between plants and microbes, in male fertility of some species, in defence as antimicrobial agents and feeding deterrents, and in UV protection.

Flavonoids also have significant activities when ingested by animals, and there is great interest in their potential health benefits, particularly for compounds such as isoflavonoids, which have been linked to anticancer benefits, and stilbenes that are believed to contribute to reduced heart disease. Condensed tannins which are plant polyphenols with protein-precipitating and antioxidant properties are involved in protein binding, metal chelation, anti-oxidation, and UV-light absorption. As a result condensed tannins inhibit viruses, microorganisms, insects, fungal pathogens, and monogastric digestion. Moderate amounts of

tannins improve forage quality by disrupting protein foam and conferring protection from rumen pasture bloat. Bloat is a digestive disorder that occurs on some highly nutritious forage legumes such as alfalfa (*Medicago sativa*) and white clover (*Trifolium repens*). Moderate amounts of tannin can also reduce digestion rates in the rumen and can reduce parasitic load sufficiently to increase the titre of amino acids and small peptides in the small intestine without compromising total digestion.

The major branch pathways of flavonoid biosynthesis start with general phenylpropanoid metabolism and lead to the nine major subgroups: the colourless chalcones, aurones, isoflavonoids, flavones, flavonols, flavandiols, anthocyanins, condensed tannins, and phlobaphene pigments. The enzyme phenylalanine ammonia-lyase (PAL) of the general phenylpropanoid pathway will lead to the production of cinnamic acid. Cinnamate-4-hydroxylase (C4H) will produce p-coumaric acid which will be converted through the action of 4-coumaroyl:CoA-ligase (4CL) to the production of 4-coumaroyl-CoA and malonyl-CoA. The first committed step channelling carbon into the flavonoid biosynthesis pathway is catalysed by chalcone synthase (CHS), which uses malonyl CoA and 4-coumaryl CoA as substrates. The *Arabidopsis BANYULS* (BAN) gene encodes a dihydroflavonol 4-reductase-like protein that may be a leucoanthocyanidin reductase (also called LCR). The reaction catalysed by BAN is generally considered to be the branching point from the general flavonoid pathway to the condensed tannin biosynthesis.

While nucleic acid sequences encoding the key enzymes in the condensed tannins biosynthetic pathway CHS and BAN have been isolated for certain species of plants, there remains a need for materials useful in modifying flavonoid biosynthesis and more specifically in modifying condensed tannin biosynthesis and therewith in modifying forage quality, for example by disrupting protein foam and conferring protection from rumen pasture bloat, particularly in forage legumes and grasses, including alfalfa, medics, clovers, ryegrasses and fescues, and for methods for their use.

In one aspect, the present invention provides substantially purified or isolated nucleic acids or nucleic acid fragments encoding the key enzymes in the condensed tannins biosynthetic pathway CHS and BAN from a clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species, or functionally active fragments or variants thereof.

The present invention also provides substantially purified or isolated nucleic acids or nucleic acid fragments encoding amino acid sequences for a class of proteins which are related to CHS and BAN or functionally active fragments or variants thereof. Such proteins are referred to herein as CHS-like and BAN-like, respectively.

In a preferred embodiment of the present invention there is provided a nucleic acid or nucleic acid fragment encoding both a BAN or BAN-like polypeptide and a CHS or CHS-like polypeptide. It will be appreciated by a person skilled in the art that nucleic acid sequences encoding the BAN or BAN-like polypeptide and the CHS or CHS-like polypeptide need not be in the same reading frame in the nucleic acid or nucleic acid fragment.

The individual or simultaneous enhancement or otherwise manipulation of CHS and BAN or like gene activities in plants may enhance or otherwise alter flavonoid biosynthesis; may enhance or otherwise alter the plant capacity for protein binding, metal chelation, anti-oxidation, and UV-light absorption; may enhance or reduce or otherwise alter plant pigment production; and may enhance or otherwise alter the amount of condensed tannins contained within forage legumes and grasses, including alfalfa, medics, clovers, ryegrasses and fescues and therewith the capacity to reduce bloating by disrupting protein foam.

Methods for the manipulation of CHS and BAN or like gene activities in plants, including legumes such as clovers (*Trifolium* species), lucerne (*Medicago sativa*) and grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species) may facilitate the production of, for example, forage legumes and forage grasses and other crops with enhanced tolerance to biotic stresses such as viruses, microorganisms, insects and fungal pathogens; altered

pigmentation in flowers; forage legumes with enhanced herbage quality and bloat-safety.

The clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species may be of any suitable type, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*), alfalfa (*Medicago sativa*), Italian or annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and red fescue (*Festuca rubra*). Preferably the species is a clover or a ryegrass, more preferably white clover (*T. repens*) or perennial ryegrass (*L. perenne*). White clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) are key pasture legumes and grasses, respectively, in temperate climates throughout the world. Perennial ryegrass is also an important turf grass.

The nucleic acid or nucleic acid fragment may be of any suitable type and includes DNA (such as cDNA or genomic DNA) and RNA (such as mRNA) that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases, and combinations thereof.

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a CHS or CHS-like protein includes the nucleotide sequence shown in Figure 2 hereto; (b) complements of the sequence shown in Figure 2; (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c).

In a further preferred embodiment of this aspect of the invention, the substantially purified or isolated nucleic acid or nucleic acid fragment encoding a BAN or BAN-like protein includes the nucleotide sequence shown in Figure 6 hereto; (b) complements of the sequence shown in Figure 6 hereto; (c) sequences antisense to the sequences recited in (a) and (b); and (d) functionally active fragments and variants of the sequences recited in (a), (b) and (c).

The term "isolated" means that the material is removed from its original environment (e.g. the natural environment if it is naturally occurring). For example, a naturally occurring nucleic acid or polypeptide present in a living plant is not isolated, but the same nucleic acid or polypeptide separated from some or all of the coexisting materials in the natural system, is isolated. Such an isolated nucleic acid could be part of a vector and/or such nucleic acids could be part of a composition, and still be isolated in that such a vector or composition is not part of its natural environment. An isolated polypeptide could be part of a composition and still be isolated in that such a composition is not part of its natural environment.

The term "purified" means that the nucleic acid or polypeptide is substantially free of other nucleic acids or polypeptides

By "functionally active" in respect of a nucleotide sequence it is meant that the fragment or variant (such as an analogue, derivative or mutant) is capable of modifying flavonoid biosynthesis in a plant. Such variants include naturally occurring allelic variants and non-naturally occurring variants. Additions, deletions, substitutions and derivatizations of one or more of the nucleotides are contemplated so long as the modifications do not result in loss of functional activity of the fragment or variant. Preferably the functionally active fragment or variant has at least approximately 80% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 90% identity, most preferably at least approximately 95% identity. Such functionally active variants and fragments include, for example, those having nucleic acid changes which result in conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence. Preferably the fragment has a size of at least 30 nucleotides, more preferably at least 45 nucleotides, most preferably at least 60 nucleotides.

By "functionally active" in the context of a polypeptide it is meant that the fragment or variant has one or more of the biological properties of the proteins CHS, CHS-like, BAN and BAN-like, respectively. Additions, deletions, substitutions and derivatizations of one or more of the amino acids are contemplated so long as the modifications do not result in loss of functional activity of the fragment or

variant. Preferably the functionally active fragment or variant has at least approximately 60% identity to the relevant part of the above mentioned sequence, more preferably at least approximately 80% identity, most preferably at least approximately 90% identity. Such functionally active variants and fragments include, for example, those having conservative amino acid substitutions of one or more residues in the corresponding amino acid sequence. Preferably the fragment has a size of at least 10 amino acids, more preferably at least 15 amino acids, most preferably at least 20 amino acids.

The term "construct" as used herein refers to an artificially assembled or isolated nucleic acid molecule which includes the gene of interest. In general a construct may include the gene or genes of interest, a marker gene which in some cases can also be the gene of interest and appropriate regulatory sequences. It should be appreciated that the inclusion of regulatory sequences in a construct is optional, for example, such sequences may not be required in situations where the regulatory sequences of a host cell are to be used. The term construct includes vectors but should not be seen as being limited thereto.

The term "vector" as used herein encompasses both cloning and expression vectors. Vectors are often recombinant molecules containing nucleic acid molecules from several sources.

By "operatively linked" is meant that said regulatory element is capable of causing expression of said nucleic acid or nucleic acid fragment in a plant cell and said terminator is capable of terminating expression of said nucleic acid or nucleic acid fragment in a plant cell. Preferably, said regulatory element is upstream of said nucleic acid or nucleic acid fragment and said terminator is downstream of said nucleic acid or nucleic acid fragment.

By "an effective amount" it is meant an amount sufficient to result in an identifiable phenotypic trait in said plant, or a plant, plant seed or other plant part derived therefrom. Such amounts can be readily determined by an appropriately skilled person, taking into account the type of plant, the route of administration and other relevant factors. Such a person will readily be able to determine a suitable

amount and method of administration. See, for example, Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, the entire disclosure of which is incorporated herein by reference.

It will also be understood that the term "comprises" (or its grammatical variants) as used in this specification is equivalent to the term "includes" and should not be taken as excluding the presence of other elements or features.

Genes encoding other CHS or CHS-like and BAN or BAN-like proteins, either as cDNAs or genomic DNAs, may be isolated directly by using all or a portion of the nucleic acids or nucleic acid fragments of the present invention as hybridisation probes to screen libraries from the desired plant employing a methodology known to those skilled in the art. Specific oligonucleotide probes based upon the nucleic acid sequences of the present invention may be designed and synthesized by methods known in the art. Moreover, the entire sequences may be used directly to synthesize DNA probes by methods known to the skilled artisan such as random primer DNA labelling, nick translation, or end-labelling techniques, or RNA probes using available *in vitro* transcription systems. In addition, specific primers may be designed and used to amplify a part or all of the sequences of the present invention. The resulting amplification products may be labelled directly during amplification reactions or labelled after amplification reactions, and used as probes to isolate full-length cDNA or genomic fragments under conditions of appropriate stringency.

In addition, short segments of the nucleic acids or nucleic acid fragments of the present invention may be used in protocols to amplify longer nucleic acids or nucleic acid fragments encoding homologous genes from DNA or RNA. For example, polymerase chain reaction may be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the nucleic acid sequences of the present invention, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding plant genes. Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, those skilled in the art can follow the RACE protocol [Frohman *et al.*

(1988), *Proc. Natl. Acad. Sci. USA* 85:8998, the entire disclosure of which is incorporated herein by reference] to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Using commercially available 3' RACE and 5' RACE systems (BRL), specific 3' or 5' cDNA fragments may be isolated [Ohara *et al.* (1989), *Proc. Natl. Acad. Sci. USA* 86:5673; Loh *et al.* (1989), *Science* 243:217, the entire disclosures of which are incorporated herein by reference]. Products generated by the 3' and 5' RACE procedures may be combined to generate full-length cDNAs.

In a second aspect of the present invention there is provided a substantially purified or isolated polypeptide from a clover, (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species, selected from the group consisting of CHS and CHS-like or BAN and BAN-like proteins and functionally active fragments and variants thereof.

The clover (*Trifolium*), medic (*Medicago*), ryegrass (*Lolium*) or fescue (*Festuca*) species may be of any suitable type, including white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*), alfalfa (*Medicago sativa*), Italian or annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*), tall fescue (*Festuca arundinacea*), meadow fescue (*Festuca pratensis*) and red fescue (*Festuca rubra*). Preferably the species is a clover or a ryegrass, more preferably white clover (*T. repens*) or perennial ryegrass (*L. perenne*).

In a preferred embodiment of this aspect of the invention, the substantially purified or isolated CHS or CHS-like polypeptide includes an amino acid sequence shown in Figure 3 hereto, and functionally active fragments and variants thereof.

In a further preferred embodiment of this aspect of the invention, the substantially purified or isolated BAN or BAN-like polypeptide includes an amino acid sequence shown in Figure 7 hereto, and functionally active fragments and variants thereof.

In a further embodiment of this aspect of the invention, there is provided a polypeptide recombinantly produced from a nucleic acid or nucleic acid fragment according to the present invention. Techniques for recombinantly producing polypeptides are well known to those skilled in the art.

Availability of the nucleotide sequences of the present invention and deduced amino acid sequences facilitates immunological screening of cDNA expression libraries. Synthetic peptides representing portions of the instant amino acid sequences may be synthesized. These peptides may be used to immunise animals to produce polyclonal or monoclonal antibodies with specificity for peptides and/or proteins including the amino acid sequences. These antibodies may be then used to screen cDNA expression libraries to isolate full-length cDNA clones of interest.

In a still further aspect of the present invention there is provided a construct including a nucleic acid or nucleic acid fragment according to the present invention.

In a still further aspect of the present invention there is provided a vector including a nucleic acid or nucleic acid fragment according to the present invention.

In a preferred embodiment of this aspect of the invention, the vector may include one or several of the following: a regulatory element such as a promoter, a nucleic acid or nucleic acid fragment according to the present invention and a terminator; said regulatory element or regulatory elements, nucleic acid or nucleic acid fragment or nucleic acids or nucleic acid fragments and terminator or terminators being operatively linked. The vector may contain nucleic acids or nucleic acid fragments encoding both CHS or CHS-like and BAN or BAN-like polypeptides, operatively linked to a regulatory element or regulatory elements, such that both CHS or CHS-like and BAN or BAN-like proteins are expressed.

The vector may be of any suitable type and may be viral or non-viral. The vector may be an expression vector. Such vectors include chromosomal, non-

chromosomal and synthetic nucleic acid sequences, e.g. derivatives of plant viruses; bacterial plasmids; derivatives of the Ti plasmid from *Agrobacterium tumefaciens*, derivatives of the Ri plasmid from *Agrobacterium rhizogenes*; phage DNA; yeast artificial chromosomes; bacterial artificial chromosomes; binary bacterial artificial chromosomes; vectors derived from combinations of plasmids and phage DNA. However, any other vector may be used as long as it is replicable, integrative or viable in the plant cell.

The regulatory element and terminator may be of any suitable type and may be endogenous to the target plant cell or may be exogenous, provided that they are functional in the target plant cell.

Preferably the regulatory element is a promoter. A variety of promoters which may be employed in the vectors of the present invention are well known to those skilled in the art. Factors influencing the choice of promoter include the desired tissue specificity of the vector, and whether constitutive or inducible expression is desired and the nature of the plant cell to be transformed (e.g. monocotyledon or dicotyledon). Particularly suitable promoters include but are not limited to the constitutive Cauliflower Mosaic Virus 35S (CaMV 35S) promoter and derivatives thereof, the maize Ubiquitin promoter, and the rice Actin promoter; and the tissue-specific Arabidopsis small subunit (ASSU) promoter,

A variety of terminators which may be employed in the vectors of the present invention are also well known to those skilled in the art. The terminator may be from the same gene as the promoter sequence or a different gene. Particularly suitable terminators are polyadenylation signals, such as the CaMV 35S polyA and other terminators from the nopaline synthase (*nos*), the octopine synthase (*ocs*) and the rbcS genes.

The vector, in addition to the regulatory element, the nucleic acid or nucleic acid fragment of the present invention and the terminator, may include further elements necessary for expression of the nucleic acid or nucleic acid fragment, in different combinations, for example vector backbone, origin of replication (ori), multiple cloning sites, recognition sites for recombination events, spacer

sequences, enhancers, introns (such as the maize Ubiquitin *Ubi* intron), antibiotic resistance genes and other selectable marker genes [such as the neomycin phosphotransferase (*npt2*) gene, the hygromycin phosphotransferase (*hph*) gene, the phosphinotricin acetyltransferase (*bar* or *pat*) gene and the gentamycin acetyl transferase (*aacC1*) gene], and reporter genes [such as beta-glucuronidase (GUS) gene (*gusA*) and green fluorescent protein (gfp)]. The vector may also contain a ribosome binding site for translation initiation. The vector may also include appropriate sequences for amplifying expression.

As an alternative to use of a selectable marker gene to provide a phenotypic trait for selection of transformed host cells, the presence of the vector in transformed cells may be determined by other techniques well known in the art, such as PCR (polymerase chain reaction), Southern blot hybridisation analysis, histochemical GUS assays, visual examination including microscopic examination of fluorescence emitted by gfp, northern and Western blot hybridisation analyses.

Those skilled in the art will appreciate that the various components of the vector are operatively linked, so as to result in expression of said nucleic acid or nucleic acid fragment. Techniques for operatively linking the components of the vector of the present invention are well known to those skilled in the art. Such techniques include the use of linkers, such as synthetic linkers, for example including one or more restriction enzyme sites.

The vectors of the present invention may be incorporated into a variety of plants, including monocotyledons (such as grasses from the genera *Lolium*, *Festuca*, *Paspalum*, *Pennisetum*, *Panicum* and other forage and turfgrasses, corn, oat, sugarcane, wheat and barley), dicotyledons (such as *Arabidopsis*, tobacco, clovers, medics, eucalyptus, potato, sugarbeet, canola, soybean, chickpea) and gymnosperms. In a preferred embodiment, the vectors may be used to transform monocotyledons, preferably grass species such as ryegrasses (*Lolium* species) and fescues (*Festuca* species), more preferably perennial ryegrass, including forage- and turf-type cultivars. In an alternate preferred embodiment, the vectors may be used to transform dicotyledons, preferably forage legume species such as clovers (*Trifolium* species) and medics (*Medicago* species), more preferably white

clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*). Clovers, alfalfa and medics are key pasture legumes in temperate climates throughout the world.

Techniques for incorporating the vectors of the present invention into plant cells (for example by transduction, transfection or transformation) are known to those skilled in the art. Such techniques include *Agrobacterium*-mediated introduction, electroporation to tissues, cells and protoplasts, protoplast fusion, injection into reproductive organs, injection into immature embryos and high velocity projectile introduction to cells, tissues, calli, immature and mature embryos. The choice of technique will depend largely on the type of plant to be transformed.

In a further aspect of the present invention there is provided a method of isogenic transformation of a dicotyledonous plant, by transforming only one of each pair of cotyledons. This enables the production of pairs of transgenic plant and corresponding untransformed negative control in an otherwise isogenic genetic background for detailed functional assessment of the impact of the transgene on plant phenotype.

Cells incorporating the vectors of the present invention may be selected, as described above, and then cultured in an appropriate medium to regenerate transformed plants, using techniques well known in the art. The culture conditions, such as temperature, pH and the like, will be apparent to the person skilled in the art. The resulting plants may be reproduced, either sexually or asexually, using methods well known in the art, to produce successive generations of transformed plants.

In a further aspect of the present invention there is provided a plant cell, plant, plant seed or other plant part, including, e.g. transformed with, a vector, nucleic acid or nucleic acid fragment of the present invention.

The plant cell, plant, plant seed or other plant part may be from any suitable species, including monocotyledons, dicotyledons and gymnosperms. In a

preferred embodiment the plant cell, plant, plant seed or other plant part may be from a monocotyledon, preferably a grass species, more preferably a ryegrass (*Lolium* species) or fescue (*Festuca* species), more preferably perennial ryegrass, including both forage- and turf-type cultivars. In an alternate preferred embodiment the plant cell, plant, plant seed or other plant part may be from a dicotyledon, preferably forage legume species such as clovers (*Trifolium* species) and medics (*Medicago* species), more preferably white clover (*Trifolium repens*), red clover (*Trifolium pratense*), subterranean clover (*Trifolium subterraneum*) and alfalfa (*Medicago sativa*).

The present invention also provides a plant, plant seed or other plant part, or a plant extract derived from a plant cell of the present invention.

The present invention also provides a plant, plant seed or other plant part, or a plant extract derived from a plant of the present invention.

In a further aspect of the present invention there is provided a method of modifying condensed tannin biosynthesis; of modifying protein binding, metal chelation, anti-oxidation, and UV-light absorption; of modifying plant pigment production; of modifying plant defence to biotic stresses such as viruses, microorganisms, insects, fungal pathogens; of modifying forage quality by disrupting protein foam and conferring protection from rumen pasture bloat, said method including introducing into said plant an effective amount of a nucleic acid or nucleic acid fragment and/or a vector according to the present invention.

In a further aspect of the present invention there is provided a method of inhibiting bloat in an animal comprising providing the animal with a forage plant including a vector, nucleic acid or nucleic acid fragment according to the present invention. The animal is preferably a ruminant, including sheep, goats and cattle. The forage plant including a vector, nucleic acid or nucleic acid fragment according to the present invention may form all or part of the feed of the animal. The forage plant preferably expresses CHS or CHS-like proteins or BAN or BAN-like proteins at higher levels than the equivalent wild-type plant. More preferably,

the forage plant expresses both CHS or CHS-like proteins and BAN or BAN-like proteins at higher levels than the equivalent wild-type plant.

In a further aspect of the present invention there is provided a method for enhancing an animal's growth rate comprising providing the animal with a forage plant including a vector, nucleic acid or nucleic acid fragment according to the present invention. The animal is preferably a ruminant, including sheep, goats and cattle. The forage plant including a vector, nucleic acid or nucleic acid fragment according to the present invention may form all or part of the feed of the animal. The forage plant preferably expresses CHS or CHS-like proteins or BAN or BAN-like proteins at higher levels than the equivalent wild-type plant. More preferably, the forage plant expresses both CHS or CHS-like proteins and BAN or BAN-like proteins at higher levels than the equivalent wild-type plant.

It is anticipated that the method of enhancing an animal's growth rate according to this invention will result in an increase in lamb growth rate of approximately 10%.

Using the methods and materials of the present invention, flavonoid biosynthesis, protein binding, metal chelation, anti-oxidation, UV-light absorption, tolerance to biotic stresses such as viruses, microorganisms, insects and fungal pathogens; pigmentation in for example flowers and leaves; herbage quality and bloat-safety; isoflavonoid content leading to health benefits, may be increased or otherwise altered, for example by incorporating additional copies of a sense nucleic acid or nucleic acid fragment of the present invention. They may be decreased or otherwise altered, for example by incorporating an antisense nucleic acid or nucleic acid fragment of the present invention.

Documents cited in this specification are for reference purposes only and their inclusion is not acknowledgment that they form part of the common general knowledge in the relevant art.

The present invention will now be more fully described with reference to the accompanying Examples and drawings. It should be understood, however, that the

description following is illustrative only and should not be taken in any way as a restriction on the generality of the invention described above.

In the Figures

Figure 1 shows the plasmid map in pGEM-T Easy of TrCHS.

Figure 2 shows the nucleotide sequence of TrCHS.

Figure 3 shows the deduced amino acid sequence of TrCHS.

Figure 4 shows the plasmid map of the binary vector pPZP221:35S^2::TrCHS.

Figure 5 shows the plasmid map in pGEM-T Easy of TrBAN.

Figure 6 shows the nucleotide sequence of TrBAN.

Figure 7 shows the deduced amino acid sequence of TrBAN.

Figure 8 shows the plasmid map of the binary vector pPZP221:35S^2::TrBAN.

Figure 9 shows the plasmid map of the binary vector pPZP221:ASSU::TrBAN:35S^2::TrCHS.

EXAMPLE 1

Preparation of cDNA libraries, isolation and sequencing of cDNAs coding for CHS, CHS-like, BAN and BAN-like proteins from white clover (*Trifolium repens*)

cDNA libraries representing mRNAs from various organs and tissues of white clover (*Trifolium repens*) were prepared. The characteristics of the white clover libraries are described below (Table 1).

TABLE 1

cDNA libraries from white clover (*Trifolium repens*)

Library	Organ/Tissue
01wc	Whole seedling, light grown
02wc	Nodulated root 3, 5, 10, 14, 21 &28 day old seedling
03wc	Nodules pinched off roots of 42 day old rhizobium inoculated plants
04wc	Cut leaf and stem collected after 0, 1, 4, 6 &14 h after cutting
05wc	Inflorescences: <50% open, not fully open and fully open
06wc	Dark grown etiolated
07wc	Inflorescence – very early stages, stem elongation, < 15 petals, 15-20 petals
08wc	seed frozen at –80°C, imbibed in dark overnight at 10°C
09wc	Drought stressed plants
10wc	AMV infected leaf
11wc	WCMV infected leaf
12wc	Phosphorus starved plants
13wc	Vegetative stolon tip
14wc	stolon root initials
15wc	Senescing stolon
16wc	Senescing leaf

The cDNA libraries may be prepared by any of many methods available. For example, total RNA may be isolated using the Trizol method (Gibco-BRL, USA) or the RNeasy Plant Mini kit (Qiagen, Germany), following the manufacturers' instructions. cDNAs may be generated using the SMART PCR cDNA synthesis kit (Clontech, USA), cDNAs may be amplified by long distance polymerase chain reaction using the Advantage 2 PCR Enzyme system (Clontech, USA), cDNAs may be cleaned using the GeneClean spin column (Bio 101, USA), tailed and size fractionated, according to the protocol provided by Clontech. The cDNAs may be introduced into the pGEM-T Easy Vector system 1 (Promega,

USA) according to the protocol provided by Promega. The cDNAs in the pGEM-T Easy plasmid vector are transfected into *Escherichia coli* Epicurean coli XL10-Gold ultra competent cells (Stratagene, USA) according to the protocol provided by Stratagene.

Alternatively, the cDNAs may be introduced into plasmid vectors for first preparing the cDNA libraries in Uni-ZAP XR vectors according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA, USA). The Uni-ZAP XR libraries are converted into plasmid libraries according to the protocol provided by Stratagene. Upon conversion, cDNA inserts will be contained in the plasmid vector pBlueScript. In addition, the cDNAs may be introduced directly into precut pBlueScript II SK(+) vectors (Stratagene) using T4 DNA ligase (New England Biolabs), followed by transfection into *E. coli* DH10B cells according to the manufacturer's protocol (GIBCO BRL Products).

Once the cDNA inserts are in plasmid vectors, plasmid DNAs are prepared from randomly picked bacterial colonies containing recombinant plasmids, or the insert cDNA sequences are amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Plasmid DNA preparation may be performed robotically using the Qiagen QiaPrep Turbo kit (Qiagen, Germany) according to the protocol provided by Qiagen. Amplified insert DNAs are sequenced in dye-terminator sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"). The resulting ESTs are analysed using an Applied Biosystems ABI 3700 sequence analyser.

EXAMPLE 2

DNA sequence analyses

The cDNA clones encoding CHS, CHS-like, BAN and BAN-like proteins were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul *et al.* (1993), *J. Mol. Biol.* 215:403-410) searches. The cDNA sequences obtained were analysed for similarity to all publicly available DNA sequences contained in the eBioinformatics nucleotide database using the BLASTN algorithm provided by

the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the SWISS-PROT protein sequence database using BLASTx algorithm (v 2.0.1) (Gish and States (1993), *Nature Genetics* 3:266-272) provided by the NCBI.

The cDNA sequences obtained and identified were then used to identify additional identical and/or overlapping cDNA sequences generated using the BLASTN algorithm. The identical and/or overlapping sequences were subjected to a multiple alignment using the CLUSTALw algorithm, and to generate a consensus contig sequence derived from this multiple sequence alignment. The consensus contig sequence was then used as a query for a search against the SWISS-PROT protein sequence database using the BLASTx algorithm to confirm the initial identification.

EXAMPLE 3

Identification and full-length sequencing of cDNAs encoding white clover CHS and BAN proteins

To fully characterise for the purposes of the generation of probes for hybridisation experiments and the generation of transformation vectors, a set of cDNAs encoding white clover CHS and BAN proteins was identified and fully sequenced.

Full-length cDNAs were identified from our EST sequence database using relevant published sequences (NCBI databank) as queries for BLAST searches. Full-length cDNAs were identified by alignment of the query and hit sequences using Sequencher (Gene Codes Corp., Ann Arbor, MI 48108, USA). The original plasmid was then used to transform chemically competent XL-1 cells (prepared in-house, $CaCl_2$ protocol). After colony PCR (using HotStarTaq, Qiagen) a minimum of three PCR-positive colonies per transformation were picked for initial sequencing with M13F and M13R primers. The resulting sequences were aligned with the original EST sequence using Sequencher to confirm identity and one of

the three clones was picked for full-length sequencing, usually the one with the best initial sequencing result.

Sequencing of TrBAN could be completed with M13F and M13R primers. Sequencing of TrCHS was completed by primer walking, i.e. oligonucleotide primers were designed to the initial sequence and used for further sequencing. The sequences of the oligonucleotide primers are shown in Table 2.

Contigs were then assembled in Sequencher. The contigs include the sequences of the SMART primers used to generate the initial cDNA library as well as pGEM-T Easy vector sequence up to the EcoRI cut site both at the 5' and 3' end.

Plasmid maps and the full cDNA sequences of white clover CHS and BAN proteins were obtained (Figures 1, 2, 5 and 6).

TABLE 2

List of primers used for sequencing of the full-length TrCHS cDNA

gene name	clone ID	sequencing primer	primer sequence (5'>3')
TrCHS	05wc1RsB06	05wc1RsB06.f1	AGGAGGCTGCAGTCAAGG
		05wc1RsB06.f2	TGCCTGAAATTGAGAAACC
		05wc1RsB06.f3	AAAGCTAGCCTTGAAGCC

EXAMPLE 4

Development of binary transformation vectors containing chimeric genes with cDNA sequences from white clover TrBAN and TrCHS

To alter the expression of the proteins involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality and bloat-safety, a set of sense and antisense binary transformation vectors was produced.

cDNA fragments were generated by high fidelity PCR using the original pGEM-T Easy plasmid cDNA as a template. The primers used (Table 3) contained recognition sites for appropriate restriction enzymes, for example EcoRI and XbaI, for directional and non-directional cloning into the target vector. After PCR amplification and restriction digest with the appropriate restriction enzyme (usually XbaI), the cDNA fragments were cloned into the corresponding site in a modified pPZP binary vector (Hajdukiewicz *et al.*, 1994). The pPZP221 vector was modified to contain the $35S^2$ cassette from pKYLX71:$35S^2$ as follows: pKYLX71:$35S^2$ was cut with ClaI. The 5' overhang was filled in using Klenow and the blunt end was A-tailed with Taq polymerase. After cutting with EcoRI, the 2kb fragment with an EcoRI-compatible and a 3'-A tail was gel-purified. pPZP221 was cut with HindIII and the resulting 5' overhang filled in and T-tailed with Taq polymerase. The remainder of the original pPZP221 multi-cloning site was removed by digestion with EcoRI, and the expression cassette cloned into the EcoRI site and the 3' T overhang restoring the HindIII site. This binary vector contains between the left and right border the plant selectable marker gene aacC1 under the control of the 35S promoter and 35S terminator and the pKYLX71:$35S^2$-derived expression cassette with a CaMV 35S promoter with a duplicated enhancer region and an rbcS terminator.

The orientation of the constructs (sense or antisense) was checked by restriction enzyme digest and sequencing which also confirmed the correctness of the sequence. Transformation vectors containing chimeric genes using full-length open reading frame cDNAs encoding white clover TrBAN and TrCHS proteins in sense orientation under the control of the CaMV $35S^2$ promoter were generated (Figures 4 and 8).

TABLE 3

List of primers used to PCR-amplify the open reading frames

gene name	primer	primer sequence (5'->3')
TrBAN	05wc2XsG02f	GGATCCTCTAGAGCACTAGTGTGTATAAGTTTCTTGG
	05wc2XsG02r	GGATCCTCTAGACCCCCTTAGTCTTAAAATACTCG

TrCHS	05wc1RsB06f	GAATTCTAGAAGATATGGTGAGTGTAGCTG
	05wc1RsB06r	GAATTCTAGAATCACACATCTTATATAGCC
TrCHS	05wc1RsB06fG	GGGGACAAGTTTGTACAAAAAAGCAGGCTTCTAGA AGATATGGTGAGTGTAGCTG
	05wc1RsB06rG	GGGGACCACTTTGTACAAGAAAGCTGGGTTCTAGA ATCACACATCTTATATAGCC

The pPZP221:35S^2 binary vector was further modified to contain two expression cassettes within one T-DNA. The expression cassette from the binary vector pWM5 consisting of the ASSU promoter and the tob terminator was PCR-amplified with Pwo DNA polymerase using oligonucleotide primers with the following sequences:

forward primer 5'-CCACCATGTTTGAAATTTATTATGTGTTTTTTTCCG-3';
reverse primer 5'-TAATCCCGGGTAAGGGCAGCCCATACAAATGAAGC-3'.

The PCR product was cut with BstXI and SmaI and cloned directionally into the equally cut pPZP221:35S^2 vector. Additionally, a GATEWAY™ cloning cassette (Invitrogen) was introduced into the multicloning site in the 35S^2:rbcS expression cassette following the manufacturer's protocol. TrBAN was cloned into the ASSU:tob expression cassette, TrCHS was amplified with the GATEWAY-compatible primers (see Table 3) and cloned into the 35S2:rbcS expression cassette. A transformation vector containing chimeric genes using full-length open reading frame cDNAs encoding white clover TrBAN protein in sense orientation under the control of the ASSU promoter and TrCHS protein in sense orientation under the control of the CaMV 35S^2 promoter within the same T-DNA was generated (Figure 9).

EXAMPLE 6

Production by isogenic transformation and analysis of transgenic white clover plants carrying chimeric white clover TrBAN and CHS genes involved in condensed tannin biosynthesis

A set of transgenic white clover plants carrying chimeric white clover genes involved in flavonoid biosynthesis, and more specifically condensed tannin biosynthesis to improve herbage quality and bloat-safety, were produced.

pPZP221:35S2::TrBAN, pPZP221:35S2::TrCHS and pPZP221:ASSU::TrBAN:35S2::TrCHS transformation vectors with TrBAN and TrCHS cDNAs comprising the full open reading frame sequences in sense orientation were generated as detailed in Example 5.

Agrobacterium-mediated gene transfer experiments were performed using these transformation vectors.

The production of transgenic white clover plants carrying the white clover TrBAN and TrCHS cDNAs, either singly or in combination, is described here in detail.

Agrobacterium tumefaciens strain AGL-1 transformed with one of the binary vector constructs detailed in Example 6 were streaked on LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown at 27 °C for 48 hours. A single colony was used to inoculate 5 ml of LB medium containing 50 µg/ml rifampicin and 50 µg/ml kanamycin and grown over night at 27 °C and 250 rpm on an orbital shaker. The overnight culture was used as an inoculum for 500 ml of LB medium containing 50 µg/ml kanamycin only. Incubation was over night at 27 °C and 250 rpm on an orbital shaker in a 2 l Erlenmeyer flask.

1 spoon of seeds (ca. 500) was placed into a 280 µm mesh size sieve and washed for 5 min under running tap water, taking care not to wash seeds out of sieve. In a laminar flow hood, seeds were transferred with the spoon into an autoclaved 100 ml plastic culture vessel. A magnetic stirrer (wiped with 70% EtOH) and ca. 30 ml

70% EtOH were added, and the seeds were stirred for 5 min. The EtOH was discarded and replaced by 50 ml 1.5% sodium hypochlorite. The seeds were stirred for an additional 45 - 60 min on a magnetic stirrer. The sodium hypochlorite was then discarded and the seeds rinsed 3 to 4 times with autoclaved ddH_2O. Finally 30 ml of ddH_2O were added, and seeds incubated over night at 10 - 15°C in an incubator.

The seed coat and endosperm layer of the white clover seeds prepared as above were removed with a pair of 18 G or 21 G needles. The cotyledons were cut from the hypocotyl leaving a ca. 1.5 mm piece of the cotyledon stalk. One of the pair of cotyledons was not transformed and transferred straight to its grid position on a 20 cm petri dish containing regeneration medium. The other cotyledon was transferred to a well on the 96 well plate containing ddH_2O. After finishing the isolation of clover cotyledons, ddH_2O in the wells was replaced with *Agrobacterium* suspension (diluted to an OD_{600} = 0.2 - 0.4). The 96 well plate was sealed with its lid and incubated for 40 min at room temperature.

After the incubation period, each cotyledon was transferred to paper towel using the small dissection needle, dried and plated onto regeneration medium RM73. The plates were then incubated at 25°C with a 16h light/8h dark photoperiod. On day 4, the explants were transferred to fresh regeneration medium. Cotyledons transformed with *Agrobacterium* were transferred to RM73 containing cefotaxime (antibacterial agent) and gentamycin. The dishes were sealed with Parafilm and incubated at 25°C under a 16/8 h photoperiod. Explants were subcultured every three weeks for a total of nine weeks onto fresh RM 73 containing cefotaxime and gentamycin. Shoots with a green base were then transferred to root-inducing medium RIM. Roots developed after 1 – 3 weeks, and plantlets were transferred to soil when the roots were well established. The transformed white clover plants then grew in a manner suitable for subsequent use, including as an animal feed.

3 – 4 leaves of white clover plants regenerated on selective medium were harvested and freeze-dried. The tissue was homogenised on a Retsch MM300 mixer mill, then centrifuged for 10 min at 1700xg to collect cell debris. Genomic DNA was isolated from the supernatant using Wizard Magnetic 96 DNA Plant System kits (Promega) on a Biomek FX (Beckman Coulter). 5 µl of the sample (50

µl) were then analysed on an agarose gel to check the yield and the quality of the genomic DNA.

Genomic DNA was analysed for the presence of the transgene by real-time PCR using SYBR Green chemistry. PCR primer pairs (Table 4) were designed using MacVector (Accelrys). The forward primer was located within the $35S^2$ promoter region and the reverse primer within the transgene to amplify products of approximately 150 - 250 bp as recommended. The positioning of the forward primer within the $35S^2$ promoter region guaranteed that homologous genes in Arabidopsis were not detected.

5 µl of each genomic DNA sample was run in a 50 µl PCR reaction including SYBR Green on an ABI (Applied Biosystems) together with samples containing DNA isolated from wild type *Arabidopsis* plants (negative control), samples containing buffer instead of DNA (buffer control) and samples containing the plasmid used for transformation (positive plasmid control).

Plants were obtained after transformation with all chimeric constructs and selection on medium containing gentamycin.

TABLE 4

List of primers used for Real-time PCR analysis of white clover plants transformed with chimeric white clover genes involved in condensed tannin biosynthesis

construct	primer 1 (forward)	primer 2 (reverse)
pPZP221TrBAN	TTGGAGAGGACACGCTGAAATC	GCAACAAAACCAGTGCCACC
pPZP221TrCHS	CATTTCATTTGGAGAGGACACGC	AACACGGTTTGGTGGATTTGC

Finally, it is to be understood that various alterations, modifications and/or additions may be made without departing from the spirit of the present invention as outlined herein.

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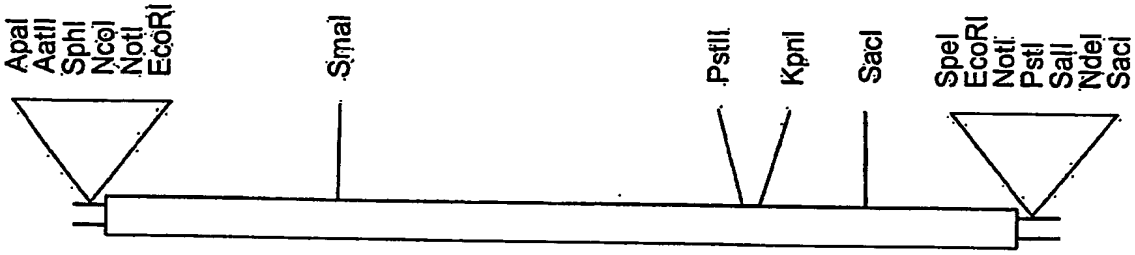
14 April 2003

FIGURES

Figure 1



TrCHS

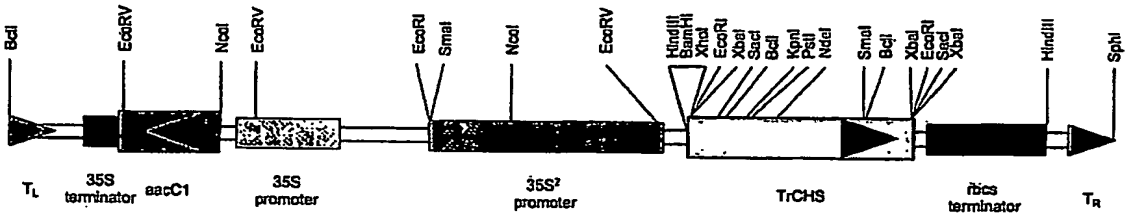
Figure 2

```
   1 GAATTCACTA GTGATTAAGC AGTGGTAACA ACGCAGAGTA CGCGGGGAAC
  51 AAAAACAACT ACGCATATTA TATATATATA TATATAGTCT ATAATTGAAA
 101 GAAACTGCTA AAGATATTAT TAAGATATGG TGAGTGTAGC TGAAATTCGC
 151 AAGGCTCAGA GGGCTGAAGG CCCTGCAACC ATTTTGGCCA TTGGCACTGC
 201 AAATCCACCA AACCGTGTTG AGCAGAGCAC ATATCCTGAT TTCTACTTCA
 251 AAATTACAAA CAGTGAGCAC AAGACTGAGC TCAAAGAGAA GTTCCAACGC
 301 ATGTGTGACA AATCCATGAT CAAGAGCAGA TACATGTATC TAACAGAAGA
 351 GATTTTGAAA GAAAATCCTA GTCTTTGTGA ATACATGGCA CCTTCATTGG
 401 ATGCTAGGCA AGACATGGTG GTGGTTGAGG TACCTAGACT TGGGAAGGAG
 451 GCTGCAGTCA AGGCCATTAA AGAATGGGGT CAACCAAAGT CAAAGATTAC
 501 TCACTTAATC TTTTGCACCA CAAGTGGTGT TGACATGCCT GGTGCTGATT
 551 ACCAACTCAC AAAACTCTTA GGTCTTCGCC CATATGTGAA AAGGTATATG
 601 ATGTACCAAC AAGGTTGTTT TGCAGGAGGC ACGGTGCTTC GTTTGGCAAA
 651 AGATTTGGCC GAGAACAACA AAGGTGCTCG TGTGCTAGTT GTTTGTTCTG
 701 AAGTCACCGC AGTCACATTT CGCGGCCCCA GTGATACTCA CTTGGACAGT
 751 CTTGTTGGAC AAGCATTGTT TGGAGATGGA GCCGCTGCAC TAATTGTTGG
 801 TTCTGATCCA GTGCCTGAAA TTGAGAAACC AATATTTGAG ATGGTTTGGA
 851 CTGCACAAAC AATTGCTCCA GACAGTGAAG GTGCCATTGA TGGTCATCTT
 901 CGTGAAGCTG GGCTAACATT TCATCTTCTT AAAGATGTTC CTGGGATTGT
 951 ATCAAAGAAC ATTAATAAAG CATTGGTTGA GGCTTTCCAA CCATTAGGAA
1001 TTTCTGACTA CAACTCAATC TTTTGGATTG CACACCCGGG TGGACCTGCA
1051 ATTCTTGATC AAGTAGAACA AAAGCTAGCC TTGAAGCCCG AAAAGATGAG
1101 GGCCACGAGG GAAGTTCTAA GTGAATATGG AAACATGTCA AGCGCATGTG
1151 TATTGTTCAT CTTAGATGAG ATGCGGAAGA AATCGGCTCA AAATGGACTT
1201 AAGACAACTG GAGAAGGACT TGATTGGGGT GTGTTGTTCG GCTTCGGACC
1251 AGGACTTACC ATTGAAACCG TTGTTCTTCG TAGCGTGGCT ATATAAGATG
1301 TGTGATTGTT TTTATTTTAA TGTATTACTT TTAATCTTGC TGCCTTGAAT
1351 TTCGATTTAA GAATAAATAA ATATATCTTT TGATAAAAAA AAAAAAAAAA
1401 AAAAAAAAAA AAGTACTCTG CGTTGTTACC ACTGCTTAAT CGAATTC
```

Figure 3

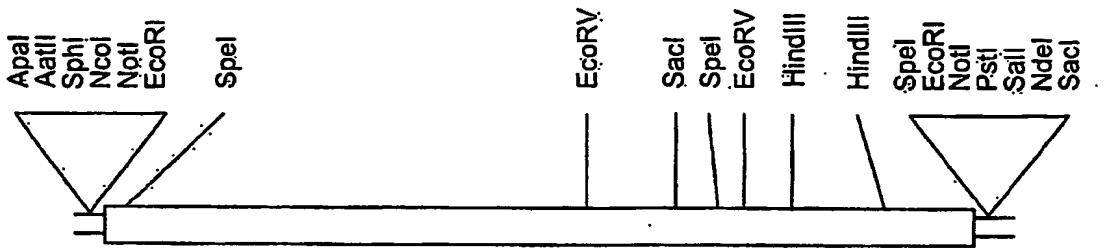
```
  1  MVSVAEIRKA QRAEGPATIL AIGTANPPNR VEQSTYPDFY FKITNSEHKT
 51  ELKEKFQRMC DKSMIKSRYM YLTEEILKEN PSLCEYMAPS LDARQDMVVV
101  EVPRLGKEAA VKAIKEWGQP KSKITHLIFC TTSGVDMPGA DYQLTKLLGL
151  RPYVKRYMMY QQGCFAGGTV LRLAKDLAEN NKGARVLVVC SEVTAVTFRG
201  PSDTHLDSLV GQALFGDGAA ALIVGSDPVP EIEKPIFEMV WTAQTIAPDS
251  EGAIDGHLRE AGLTFHLLKD VPGIVSKNIN KALVEAFQPL GISDYNSIFW
301  IAHPGGPAIL DQVEQKLALK PEKMRATREV LSEYGNMSSA CVLFILDEMR
351  KKSAQNGLKT TGEGLDWGVL FGFGPGLTIE TVVLRSVAI
```

Figure 4



pPZP221:35S²TrCHS

Figure 5



TrBAN

Figure 6

```
   1 GAATTCGATT AAGCAGTGGT AACAACGCAG AGTACGCGGG ATAAAAACTG
  51 CACTAGTGTG TATAAGTTTC TTGGTGAAAA AAGAGTTTGT AAATTAACAT
 101 CATGGCTAGT ATCAAACAAA TTGGAAACAA GAAAGCATGT GTGATTGGTG
 151 GCACTGGTTT TGTTGCATCT ATGTTGATCA AGCAGTTACT TGAAAAGGGT
 201 TATGCTGTTA ATACTACCGT TAGAGACCCA GATAGCCCTA AGAAAATATC
 251 TCACCTAGTG GCACTGCAAA GTTTGGGGGA ACTGAATCTA TTTAGAGCAG
 301 ACTTAACAGT TGAAGAAGAT TTTGATGCTC CTATAGCAGG ATGTGAACTT
 351 GTTTTTCAAC TTGCTACACC TGTGAACTTT GCTTCTCAAG ATCCTGAGAA
 401 TGACATGATA AAGCCAGCAA TCAAAGGTGT GTTGAATGTG TTGAAAGCAA
 451 TTGCAAGAGC AAAAGAAGTT AAAAGAGTTA TCTTAACATC TTCGGCAGCC
 501 GCGGTGACTA TAAATGAACT CAAAGGGACA GGTCATGTTA TGGATGAAAC
 551 CAACTGGTCT GATGTTGAAT TTCTCAACAC TGCAAAACCA CCCACTTGGG
 601 GTTATCCTGC CTCAAAAATG CTAGCTGAAA AGGCTGCATG GAAATTTGCT
 651 GAAGAAAATG ACATTGATCT AATCACTGTG ATACCTAGTT TAACAACTGG
 701 TCCTTCTCTC ACACCAGATA TCCCATCTAG TGTTGGCTTG GCAATGTCTC
 751 TAATAACAGG CAATGATTTT CTCATAAATG CTTTGAAAGG AATGCAGTTT
 801 CTGTCGGGTT CGTTATCCAT CACTCATGTT GAGGATATTT GCCGAGCTCA
 851 TATATTTCTT GCAGAGAAAG AATCAGCTTC TGGTAGATAC ATTTGCTGTG
 901 CTCACAATAC TAGTGTTCCC GAGCTTGCAA AGTTTCTCAA CAAACGATAT
 951 CCTCAGTATA AAGTTCCAAC TGAATTTGAT GATTGCCCCA GCAAGGCAAA
1001 GTTGATAATC TCTTCTGAAA AGCTTATCAA AGAAGGGTTC AGTTTCAAGC
1051 ATGGTATTGC CGAAACTTTC GACCAGACTG TCGAGTATTT TAAGACTAAG
1101 GGGGCACTGA AGAATTAGAT TTTGATATTT CTAATTCAAT AGCAAACTCT
1151 AAGCTTGTTA TGTGTTTGTG AAGTTCAGAG TGAAATATCA AATGAATAAG
1201 TGGAGAGAGC ACAATAAGAG GAGAGCACAA TAATTTTGGA AAAAAAAAAA
1251 AAAAAAAAAA AAAAAAAAGT ACTCTGCGTT GTTACCACTG CTTAATCACT
1301 AGTGAATTC
```

Figure 7

```
  1  MASIKQIGNK KACVIGGTGF VASMLIKQLL EKGYAVNTTV RDPDSPKKIS
 51  HLVALQSLGE LNLFRADLTV EEDFDAPIAG CELVFQLATP VNFASQDPEN
101  DMIKPAIKGV LNVLKAIARA KEVKRVILTS SAAAVTINEL KGTGHVMDET
151  NWSDVEFLNT AKPPTWGYPA SKMLAEKAAW KFAEENDIDL ITVIPSLTTG
201  PSLTPDIPSS VGLAMSLITG NDFLINALKG MQFLSGSLSI THVEDICRAH
251  IFLAEKESAS GRYICCAHNT SVPELAKFLN KRYPQYKVPT EFDDCPSKAK
301  LIISSEKLIK EGFSFKHGIA ETFDQTVEYF KTKGALKN
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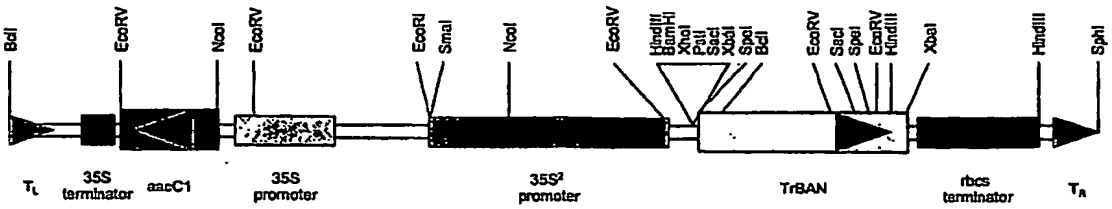
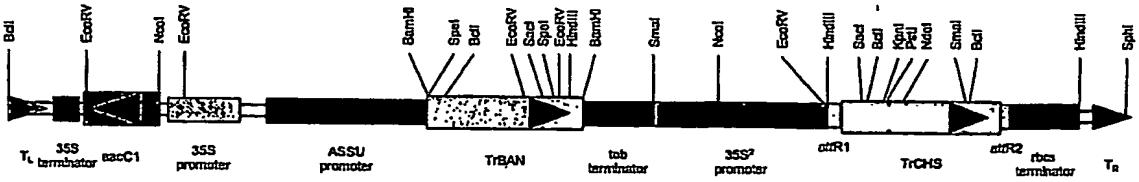
Figure 8



pPZP221:35S²TrBAN

Figure 9



pPZP221:ASSU::BAN:35S²::CHS